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(54) Methods and means for the treatment of immune related diseases such as Graft vs. Host disease

(57) The present invention provides novel means and methods for treating unwanted side reactions in transplantations, such as GvH and allograft rejection. The invention provides immunotoxines comprising an antibody and a toxic substance, whereby cocktails of such conjugates directed to different targets associated with one population of cells, wherein one target is chosen from CD3 or CD7. The preferred combination is a cocktail directed against both.

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Description

[0001] The invention relates to the field of immune system related diseases, in particular to novel means and methods for treating these diseases. More in particular the invention provides novel means for eliminating or suppressing populations of unwanted CD3 and/or CD7 positive cells.

[0002] Typically the invention finds applications in the field of allogeneic bone marrow transplantation.

[0003] Allogeneic bone marrow transplantation (BMT) is a world-wide accepted method to treat a number of severe disorders like leukemia, myelo-dysplastic syndrome, bone marrow failure, immune deficiency, storage diseases and hemoglobinopathies (1-6). For a good engraftment, the bone marrow must contain a minimum number of T-cells (7-10). These cells may also confer benefit as they contribute to the so-called graft-versus-leukemia effect which involves the elimination of residual malignant cells (11-13). However, donor T cells may react with normal tissues of the host causing graft-versus-host-disease (GVHD) which results in serious damage to the skin, liver and gastro-intestinal tract (2,14-16). When this disease occurs within the first three months after BMT, it is classified as acute GVHD and when it develops at a later stage it is referred to as chronic GVHD. The severity of the clinical symptoms is expressed in four grades, grade I referring to minimal GVHD, and grade IV to the most severe form which is usually fatal and involves epidermiolysis, liver failure, and severe diarrhea (14).

[0004] The incidence and severity of GVHD can be diminished by depletion of T-cells from the graft. At the University Hospital Nijmegen, 98% of lymphocytes are depleted from the graft using counterflow centrifugation (17). However, despite depletion of the vast majority of T cells, GVHD can still occur. The annual incidence of grade II-through IV GVHD at the University Hospital Nijmegen is ~ 6 out of a total of 40 allogeneic bone marrow transplant recipients. World-wide GVHD occurs in 30-70% of HLA-matched recipients and contributes to death in 20-40% of those affected (2,18). Even if a patient survives severe GVHD, this results in long-lasting disability and morbidity leading to repeated admission to hospital. The development of an effective alternative treatment for GVHD will therefore have a major impact on both survival and the quality of life of allogeneic BMT recipients.

[0005] Current treatment is usually as follows.

[0006] As immuno-prophylaxis cyclosporin is administered intravenously from one day before transplantation onwards (3 mg/kg/d for 15 days, and thereafter 2 mg/kg/d). As soon as the patient can take oral medication, cyclosporin is given orally (6 mg/kg/d). If the patient develops GVHD, 1st-line therapy in the form of corticosteroids is given (prednison: 1 mg/kg/d). In case the patient does not respond to this therapy within 48 hours, or if the GVHD is progressive within 24 hours, high-dose methylprednisolon (Solumedrol 4 x 250 mg/day) is given as second line therapy. When high-dose methylprednisolon has failed, patients are currently treated with Leukotac (a-IL2-receptor MoAb) (19). Although responses are seen in some cases during administration of Leukotac, GVHD normally recurs once treatment is discontinued. GVHD will eventually be fatal for about 90% of the patients not responding to the second line therapy within 3-5 days.

[0007] The present invention provides novel means and methods for among others the treatment of GvHD using molecules specifically recognizing receptors on cells to be eliminated. In a number of preferred embodiments at least one such a molecule will be coupled to a toxic moiety. Such molecules are often referred to immunotoxins.

[0008] An immunotoxin (IT) is a conjugate of for instance a monoclonal antibody (MoAb), usually developed in a mouse, coupled to a potent toxin such as the A-chain of the potent plant toxin ricin (RTA) (figure 1).

[0009] The mechanism of action of the IT is depicted in figure 2. The MoAb-moiety first binds specifically to an antigen expressed on those cells which are to be eliminated, wherafter the entire antigen-IT complex is internalized. Once inside the cell, the bond between the MoAb and the toxin is broken thereby releasing free toxin into the cytoplasm where it inhibits protein synthesis irreversibly by means of a catalytic reaction which culminates in the cell's death. Since the toxin-moiety is incapable of entering the cell autonomously and is inactive outside the cell, ITs are only hazardous to cells that express the specific target antigen and are capable of internalising the IT complex.

[0010] Since their introduction in 1979, ITs have been used to treat a variety of diseases including solid and diffuse tumors, immunological disorders and viral infections. At the Department of Hematology of the University Hospital Nijmegen, ITs have been investigated since 1984 within the framework of bone marrow transplantation to eliminate malignant T/B-cells from autologous bone marrow grafts and normal T cells from allogeneic bone marrow grafts (21-31).

[0011] Clinical results published so far are mainly from Phase I/II studies. The commonly observed Phase I dose-limiting toxicities of RTA-ITs are vascular leak syndrome (VLS) and myalgias. These side effects, due to the 'bystander' effects of the toxin, appeared to be transient and generally ceased shortly after immunotoxin administration was discontinued (32). Two different trials witnessed toxicity due to crossreactivity of the MoAb-part with undesired tissue (33,34). In both cases the IT, directed against ovarian carcinoma and breast carcinoma, respectively, crossreacted with neural tissue resulting in severe neurotoxicity. These observations stress the importance of extensive preclinical screening for crossreactivity with normal tissue. A common feature in most clinical trials was the development of human antibodies against both the MoAb and the toxin part of the IT (32). These humoral immune responses were not associated with

enhanced toxicity nor allergic reactions, but decreased the serum half life of the IT. Nevertheless, clinical responses were seen even in the face of anti-IT antibody (35-37).

[0012] The efficacy of treatment with ITs, as observed in the Phase I trials, has shown to be strongly influenced by the accessibility of target cells. So far, treating solid tumors has met with little success whereas treatment of disorders like lymphomas and certain immunological diseases resulted in partial and complete responses in 12-75% of cases (38). These results are impressive considering the fact that treatment of cancer with >90% of the drugs currently available produced fewer than 5% of partial and complete responses in Phase I trials (38).

[0013] Thus the present invention provides a pharmaceutical composition for eliminating or reducing the number of unwanted CD3 and/or CD7 positive cells, comprising a mixture of at least a first molecule specifically recognizing CD3 or CD7 and at least a second molecule specifically recognizing another ligand receptor associated with the surface of such an unwanted cell, whereby at least one of the specifically recognizing molecules is provided with a toxic moiety. A pharmaceutical composition is defined herein as any composition which can be administered to an individual, be it as one single dosis or as a regimen of doses by any viable route, preferably by intravenous administration, optionally containing usual vehicles for administration and/or components of regular treatment of the relevant immune related disease. Unwanted cells are any cells that comprise CD3 and/or CD7 (and of course many other) molecules associated with the cell surface, which cells are involved in a pathological condition in an individual. Typically these cells are T-cells or NK-cells or other cells playing a role in GvH or allograft rejection. Also aberrant cells (T cell leukemias or lymphomas for instance) comprising CD3 and/or CD7 (preferably both) can be eliminated or suppressed according to the invention.

[0014] A molecule specifically recognizing CD3 or CD7 or another ligand-receptor is a term well understood in the art. It means any molecule having a relatively high binding affinity and specificity for CD3, CD7 or the receptor. It may typically be a ligand for a receptor or an antibody for either CD3, CD7 or another receptor (receptor is defined as any molecule capable of a specific interaction), which antibody may be truncated or humanized or altered in any other way without losing its specificity (such alterations are herein defined as derivatives and/or fragments). Toxic moieties are basically any and all molecules that lead to toxicity for the target cell be it directly or indirectly, thus including but not limited to lectins, ricin, abrin, PE toxin, diphteria toxin, radio-isotopes, cytostatic drugs such as adriamycin, apoptosis inducing agents and prodrug converting substances together with prodrugs such as the thymidin kinase and gancyclovir combination. Preferred is Ricin A, preferably deglycosylated (In order to prevent the binding of ricin A to carbohydrate-receptors expressed by liver cells, deglycosylated ricin A (dgRTA) (20) is preferred). In prodrug embodiments typically one of the molecules specifically recognizing CD3 or CD7 or a receptor may be provided with the converting agent and the other with the prodrug. CD3 however does not need to be coupled to a toxic moiety to have an effect, since it blocks the interaction of the T cell receptor with APC's. This is one of the advantages of the present invention which are not poresent in the prior art.

[0015] As stated above it is preferred that both CD3 and CD7 are targeted by specific binding molecules therefor. This cocktail has proven itself in preliminary clinical studies, in an embodiment where the toxic moiety is attached to both specifically binding molecules, which are both antibodies. A surprising effect of this cocktail is that although in some cases GvH relapse occurs, this relapse is now treatable with low dose corticosteroids, which in first instance the GvH was not. Thus such a combination is another preferred embodiment of the present invention. The invention thus also provides a pharmaceutical composition as described above, whereby said first molecule specifically recognizes CD3 and said second molecule specifically recognizes CD7.

[0016] The toxic moiety may be coupled to the specifically binding molecule in any manner, such as by making a fusion protein by recombinant means, typically including a protease cutting site between binding molecule and toxic (protein) moiety, but for ease of manufacturing and freedom of choice in toxic moieties chemical coupling is preferred, optionally by an acid-labile linker. (Upon internalization a conjugate typically goes through a lysosome).

[0017] The invention also provides a pharmaceutical composition according wherein at least two molecules specifically recognizing different receptors are provided with toxic moieties, which may be the same or different toxic moieties. There is a major advantage to using different toxic moieties when the side effects of the moieties are different, because higher doses can then be given. Typically the pharmaceutical compositions according to the invention may further comprise at least one further molecule specifically recognizing CD5, CD2, CD4, CD8 or an IL-2 receptor, which may also be coupled to a toxic moiety. This may provide higher efficacy, but may also be used to provide higher specificity for groups of cells, also in combination with for instance prodrug regimes.

[0018] It is preferred that when the molecule specifically recognizing CD3 is an antibody that the antibody is of the IgG gamma-2B class, because this antibody does not lead to complement activation in this setting. Thus the invention in yet another embodiment provides a pharmaceutical composition wherein said first molecule is a gamma2B IgG antibody or a derivative thereof, which recognizes CD3.

[0019] Doses used are given in the detailed description hereof. The limitas of doses of immunotoxins in regimens like the invented one are typically dependent on the immunotoxin, both because of the specificity and affinity of the specific binding molecules as well as because of the different tolerated doses for different drugs. Expressed in equivalents of Ricin A deglycosylated the limits will be generally within at least 25 micrograms per square meter body surface (a grown

human of 80 kg, typically has 2 square meters of surface), preferably 100 micrograms of Ricin A per square meter of body surface. This is a lower limit of what may be given in one or more doses over one or more days of treatment. Generally the total dosis of Ricin A equivalents should not be higher than 25 mg per square meter body surface.

[0020] Typically the compositions according to the invention will be used for the treatment of Graft vs. Host disease, Graft rejections, T-cell leukemias, T-cell lymphomas or other CD3 and/or CD7 positive malignancies, autoimmune diseases or infectious immune diseases such as HIV-infection. As stated hereinbefore in GvHD and graft rejection a typical effect is seen in that after treatment with a composition as disclosed herein, the relapse is treatable with low doses of corticosteroids.

[0021] Such a regimen is thus also part of the invention.

[0022] Thus the invention also provides a kit of pharmaceutical compositions for treating Graft vs. Host disease and/or graft rejection comprising a composition according to anyone of claims 1-13 and a pharmaceutical composition comprising at least one corticosteroid. The invention leads to a drop in numbers in the population of unwanted cells to at least 20 % of the original amount, usually even to 5% or less. Typically this number stays low over a prolonged period of time in contrast to what prior art regimes have accomplished. The exemplified composition not only targets T cells, but also NK cells which is another advantage of the present invention.

Detailed description of the invention

[0023] The rationale for ITs to treat GVHD is that these conjugates can be used for an efficient and specific eradicating of immunocompetent T cells responsible for the disease. In this perspective, ITs might be more effective and may cause less side effects than broadly immunosuppressive reagents as cyclosporine and corticosteroids. In 1990, Byers et al reported of a Phase I clinical trial in which they intravenously administered an anti-CD5 RTA-IT (Xomazyme-CD5) to treat corticosteroid-resistant GVHD (39). The initial results were very promising with skin, gastrointestinal tract, and liver disease responding in 73, 45, and 28% of cases, respectively (39). However, more recent clinical trials showed that Xomazyme CD5 was no more effective than alternative treatments (18). Consequently, the further development of Xomazyme-CD5 was abandoned.

[0024] Encouraged by the initial success of the IT-based treatment of GVHD, we set up to develop alternative ITs with superior anti-T cell activity. In order to achieve this, we conjugated RTA to a panel of MoAbs that react with antigens that are expressed (almost) exclusively on T cells namely, the T-cell differentiation-antigens CD3, CD5 and CD7, and have assayed each for its anti-T cell activity. From this preclinical study, it appears that a cocktail of SPV-T3a-RTA (CD3-IT) and WT1-RTA (CD7-IT) has the highest potential for treating patients with severe GVHD and that this mixture affords:

- **Synergistic cytotoxicity** in which the simultaneous incubation of half the effective individual dose of SPV-T3a-RTA and WT1-RTA is more effective in eliminating T cells than either IT alone (including the CD5-IT).
- **Broad mechanism of action** in which binding of SPV-T3a to the T-cell receptor/CD3 complex results in an additive immunosuppressive effect by blocking the recognition by the donor T cells of the foreign patient antigens. This effect is independent of action of ricin A. Moreover, the binding of this particular CD3-MoAb does not stimulate T cells to produce cytokines which would otherwise augment the severity of GVHD.
- **Broad spectrum reactivity** by which WT1-RTA is also reactive against NK-cells. These lymphocytes are thought to aggravate the severity of GVHD especially in the later phase of the disease.

2.4 Clinical history of the IT-cocktail components:

[0025] *SPV-T3a:* SPV-T3a is a mouse IgG2b MoAb directed against the human T-cell differentiation antigen CD3 (40).

[0026] Anti-CD3 antibody therapy is often associated with the cytokine release syndrome caused by the binding to the T-cell receptor/CD3 complex (41-44). One of the important benefits of SPV-T3a is that this particular MoAb does not induce cytokine release because of its IgG2b-isotype (45,46).

[0027] At the Department of Hematology of the University Hospital Nijmegen part of the bone marrow obtained from HLA-matched unrelated donors is currently treated *ex vivo* with a cocktail of SPV-T3a-RTA and WT1-RTA in order to eliminate immunocompetent T cells. The patients transplanted with this marrow showed normal hematopoetic reconstitution without any signs of toxicity (n = 3, data not shown).

[0028] *WT1:* MoAb WT1 is a mouse MoAb of IgG2a isotype directed against the human T cell differentiation antigen CD7 (47, 48).

[0029] At the Department of Nephrology of the University Hospital Nijmegen three patients which underwent a kidney-transplantation, have been treated with WT1 in order to treat an acute rejection. The administration of unconjugated WT1 appeared to be safe and did not result in allergic reactions nor in severe toxicities (Dr W Tax, personal communication). No clinical efficacy could be noted.

[0030] WT1 has been conjugated to dgRTA and administered to rhesus monkeys to test its suitability for use in the

therapy of leukemic meningitis (49). The major conclusion of this study was that WT1-dgRTA may be safely administered intrathecally to rhesus monkeys and could be a good candidate for the treatment of T-lymphoblastic CNS leukemia.

[0031] At the DePartment of Hematology of the University Hospital Nijmegen, WT1-RTA is used since 1986 for the *ex vivo* purging of autologous BM of patients suffering from high-risk T-cell leukemia/lymphoma in order to eliminate residual malignant cells. After purging, neither neutrophil engraftment nor immunological reconstitution was delayed (n = 20) (25).

[0032] ***SMPT cross-linker:*** The MoAbs are conjugated to dgRTA using the chemical cross-linker SMPT (figure 3). The cross-linker contains a disulfide bond which is important for the intracellular dissociation of the MoAb and dgRTA (necessary for toxicity, see figure 2).

[0033] SMPT is a so called 'second-generation cross-linker', characterized by having a hindered disulfide bond (due to the presence of the phenyl ring). This renders the SMPT-linker less susceptible to *extracellular* reduction by thiols present in the tissues and blood, and, therefore, results in a prolonged serum half-live of the IT. Thorpe et al demonstrated in an *in vivo* mice tumor model that using SPMT instead of the first-generation cross-linker SPDP, strongly improves the anti-tumor effect of their dgRTA-based ITs (20). Amlot et al performed a Phase I trail in which they studied the treatment of malignant lymphoma by intravenous administration of a SMPT-conjugated IT (RFB4[IgG]-dgRTA) (50) Due to the long serum half-live of 7.8 hours, therapeutic serum concentrations could be maintained between the infusions given at 48-hours intervals.

[0034] ***dgRTA:*** The earliest RTA-based ITs consisted of a MoAb conjugated to native RTA. The oligosaccharides present on the native RTA, resulted in rapid hepatic clearance and hepatotoxicity *in vivo* (20, 51). This problem has been addressed in the second-generation ITs which make use of either deglycosylated RTA (dgRTA) or non-glycosylated recombinant ricin A (rRTA) (52, 53).

[0035] Vitetta and colleagues have reported the administration of dgRTA-based ITs to patients with refractory B-cell non-Hodgkin's lymphoma. They tested two different constructs. In the first, the Fab' fraction of MoAb RFB4 (anti-CD22) was conjugated to dgRTA (50). In the later construct they used RFB4 whole molecule (54).

[0036] The ITs were administered by 4-hour intravenous infusions given at 48-hours intervals. The Phase I dose limiting toxicities included pulmonary edema, expressive aphasia, and rhabdomyolysis with acute renal failure. Other side effects included hypoalbuminemia, weight gain, fever, tachycardia, decrease in electrocardiogram voltage, myalgias, anorexia, and nausea. The maximum tolerated dose (MTD) was 75 mg/m2 for the Fab'-dgRTA and 32 mg/m2 for whole IgG-dgRTA, which appeared to be inversely related to the serum half life (86 minutes and 7.8 hours, respectively). The two forms of the dgRTA-IT demonstrated no significant difference in clinical responses (partial and complete responses in 45% of the patients receiving > 50% of the MTD), in immunogenicity or in the toxic side effects. Because of its lower costs, the IgG-dgRTA IT was selected for further development.

[0037] The major findings to be learned from these studies are: *a)* The MTD of dgRTA-ITs is dependent primarily on the size of an individual dose rather than the cumulative dose. When administering RFB4(IgG)-dgRTA at 48-hours intervals at doses of 8 mg/m2 or less, only grade I or II toxicities were observed. Total doses of 32 mg/m2 RFB4(IgG)-dgRTA were consistently safe. As a consequence of the relative long T1/2, therapeutic serum concentrations (~ 1.8 µg/ml) could be maintained during and between infusions *b)* Side effects of the dgRTA-ITs administration were relatively modest and consisted predominantly of VLS and myalgia. No hepatotoxicity and minimal BM toxicity was observed. *c)* Patients with underlying pulmonary disease should not be treated because of the danger of VLS contributing to further pulmonary insufficiency.

[0038] Patients received four doses of IT-cocktail administered intravenously in 4-hours infusions at 48-hours intervals. If no clinical response is observed and if no severe toxicities (grade III or IV) occur, the study is continued with the next higher protein dose level.

4.0 Patient population

[0039] Patients have received 2nd-line high dose corticosteroid therapy (methylprednisolon 1000 mg/d) for at least three days without any decrease of the severity of aGVHD.

[0040] Patients are EXCLUDED from participation in the study if:

1. The patient has a significant history or current evidence of intrapulmonary disease.
2. The patient has history of allergy to mouse immunoglobulins or ricin.
3. The patient has circulating high levels of human anti-mouse antibodies (HAMA).

5.0 Treatment

5.1 Pharmacological information:

[0041] The IT-cocktail has been prepared by the Department of Hematology under supervision of the Department of Clinical Pharmacy of the University Hospital Nijmegen. The IT-cocktail is stored at -80°C at 1 mg/ml in 0.15 M NaCl, in lots of 5 and 20 mg. Before infusion, the IT-cocktail will be filtered through a 0.22 μm filter and diluted to a final volume of 100 ml in normal saline solution. The ID_{50} against the T cell line Jurkat is taken as the standard for biological activity when evaluating the quality of different lots of IT-cocktail.

5.2 Immunotoxin administration:

[0042] Immunotoxins are administered via a central venous catheter. Prior to therapy, patients are given an i.v. test done with 200 μg IT-cocktail. Therapy is only started in the absence of anaphylactoid reactions. The IT-cocktail is administered in four doses at 48-hours intervals. The rationale of this is to give all the IT-cocktail before any host antibody response is expected to arise (usually not before 10 to 14 days after administration of xenogenic Ig).

[0043] The patient is initially treated with two subsequent doses of 2 mg/m^2. At this dose level no side effects are observed. In the absence of grade III or IV toxicities, the dose will be enhanced to 4 mg/m^2 if necessary..

5.3 Guidelines for dose modification:

[0044] Toxicities related to the immunotoxin administration are graded as grade I (mild), II (moderate), III (severe) or IV (life threatening) based on World Health Organization (WHO) guidelines (Appendix V). Special attention must be paid to the vascular leak syndrom (VLS). The physical signs of VLS being weight gain, peripheral edema, decrease in blood pressure, hypoalbuminemia, and small pleural effusions.

[0045] ***Consecutive doses given to the same patient***: Infusion of the second, third and fourth dose at any dose level is dependent upon the toxicity achieved after the previous infusion:

Grade I toxicity: no change in the scheduled dosage.
Grade II toxicity: 24 hours-delay of the dosage, with the next dose given if toxicity improves.
Grade III toxicity: the next dosage of immunotoxin will be withheld and only given if toxic parameters have improved (halving of the dosage can be considered).
Grade IV toxicity: no further dosage.

[0046] ***Dose escalation:*** Progression from one dose level to the next should only occur after:

- The patient(s) of the group treated with the previous dose level have received four doses of IT-cocktail and have been observered for at least 48 hours after the last dosis, and,
- Dose limiting toxicity has not been reached.

[0047] ***Dose limiting toxicity:*** Dose limiting toxicity is defined as the occurence of adverse reactions of grade III or IV in an individual patient. If two patients experience a Grade III toxicity or if Grade IV toxicity occurs in a single patient, three additional patients will be entered at this dose level. If non of these additional patients demonstrate toxicity of grade III or IV, administration will again be continued to the next higher dose level. If Grade IV toxicity occurs in two patients at a given dose, the next patients will be treated with the previous dose level which will be considered the Maximum Tolerable Dose (MTD).

5.4 Concomitant medication and treatment:

[0048] Immunosuppressive agents used for prophylaxis and initial treatment may maintain unchanged throughout immunotoxin therapy.

6.1 Pretreatment studies:

[0049] Before entry into the study the patient undergoes a general examination consisting of medical history, physical examination with special emphasis on acute GVHD, measurement of oxygen saturation, electrocardiogram (ECG), and chest x-ray. The laboratory measurements will include Na+, K+, Cl-, HCO_3^-, urea, creatine, billirubin, glucose, AP, ASAT (GOT), ALAT (GPT), gGT, LDH, total protein plus electrophoresis, leukocytes plus differentiation, red cells, hemoglobin,

hematocrit, thrombocytes.

[0050] The patient is assayed for human anti-mouse and anti-ricin responses (HAMA/HARA), and serum levels of IL-2, TNF-a and IFN-g. Furthermore a quantitative alloreactive T-helper/T-cytocoxic precursor assay will be performed (see Appendix I).

6.1 Follow-up studies:

[0051] Daily complete physical examination is performed during IT-cocktail administration until 2 days after the last dose and then weekly thereafter. Blood is analyzed daily for Na+, K+, Cl-, HCO3-, urea, creatine, glucose, albumin, leukocytes plus differentiation, red cells, hemoglobin, hematocrit, and thrombocytes. Every two days for billirubin, AP, ASAT (GOT), ALAT (GPT), gGT, LDH, total protein plus electrophoresis (see Appendix I).

[0052] Vital signs (blood pressure, pulse, respiration frequention, and temperature) are checked every 15 minutes during the 1st hour post infusion, every 30 min during the 2nd up to 4th hour post infusion and from then on every hour up to eight hours post infusion. Besides, vital signs are assessed daily during IT-cocktail administration until 2 days after the last dose and then weekly thereafter.

[0053] *Pharmocokinetics and clearance of ITs:* Blood samples are collected at 0, 1, 3, 4, 8, 12, 24, and 48 hours after each infusion. The serum concentrations of SPV-T3a-dgRTA and WT1-dgRTAare quantitatively determined in a sensitive and mAb-isotype specific immuno-radiometric assay (IRMA). From these results the individual serum halflifes of the two is calculated using the non linear least squares regression analysis.

[0054] *Measurement of humoral responses:* In order to examine HAMA and HARA responses, serum samples are obtained one day pre-injection and subsequently weekly after the first infusion until the patient comes off study. The concentration of HAMA and HARA is determined in a sensitive radiometric assay.

[0055] *Immunological monitoring:* Blood is sampled every other day during IT-cocktail administration until 2 days after the last dose and then weekly thereafter for immunological monitoring. PBLs are isolated and evaluated for composition by flow cytometry using antibodies reacting specifically with T cells subsets, B-cells, monocyte/macrophages, and NK cells. Serum are collected to determine levels of IL-2, TNF-a and IFN-g by commercial enzyme-linked immunosorbent assay (ELISA) kits.

[0056] The proliverative and cytotoxic activity of allo-reactive PBLs is tested 2 days following the last dose using standard T-helper and T-cytotoxic-precursor assays, respectively.

[0057] *Staging and Grading of GVHD and clinical responses:* GVHD is scored daily during IT-cocktail administration until 2 days after the last dose and then weekly thereafter until the patient comes off study. Each organ system is staged grade 1 through 4 aGVHD according to the criteria of Glucksberg et al (Appendix II): skin by amount of surface involved with rash, gastrointestinal tract by the volume of diarrhea, and liver by serum bilirubin levels. Patients are also given an overall grade of aGVHD based on severity of organ involvement (Appendix II).

[0058] Responses to therapy are defined as follows:

- complete response (CR): the disappearance of symptoms in all organ systems;
- partial response (PR): improvement of ^{3}1 organ, with no worsening in other organs.
- mixed response (MR): improvement of ^{3}1 organ, with worsening in ^{3}1 other organ.
- stable disease: no significant change in any organ system.
- progressive disease (PD): progression in ^{3}1 organ-system without improvement in any organs.

[0059] The duration of reponse is defined as the period from the date the response was first recorded to the date on which subsequent progressive disease is first noted.

Results.

[0060] Animal toxixity studies.

[0061] Animal toxicity studies are summarized in appendix III.

Clinical study.

[0062] The results of the clinical study are reported in Appendix IV.

Materials and methods.

Making monoclonal antibodies.

[0063] Monoclonal antibodies disclosed herein are prepared using hybridoma technology well known in the art. Selection steps to slect immunoglobulins having the properties discloesd hereinabove are also well known in the art and may include affinitychromatography and the like.

Preparation of F(ab')2 fragments.

[0064] $F(ab')_2$ fragments of antibodies were prepared using a "$F(ab')_2$ preparation kit" (Pierce, Rockford, IL), according to the manufacturers' protocol. Briefly, antibodies were incubated with immobilised pepsin at pH 4.2 (20 mM sodium acetate buffer) for 4, and 16 h, respectively. Undigested IgG molecules, and Fc-fragments were removed by affinity chromatography with protein A-sepharose. Remaining fragments smaller than 30 kD were removed by means of a centriprep-30 concentrator (Amicon, Beverly, MA). The purity of $F(ab')_2$ fragments was determined by SDS-PAGE, which revealed less then 1% contamination with either intact IgG or Fc-fragments (data not shown).

IT preparation.

[0065] Antibodies were conjugated to ricin A (kindly provided by Dr. F.K. Jansen; Centre de Recherches Clin Midy, Montpellier, France) using N-succinimydyl 3-(2-pyridyldithio)propionate (SPDP; Pharmacia) or SMPT, as described. (The conjugation ratios of ricin A to mAb were estimated by measurement of absorbance at 280 nm and RIA, and were determined to be in the order of 0.8 to 1.2. Preservation of antibody-binding activity following conjugation was assessed by FCM.

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Table I

Dose levels:					
	Dosage of IT-cocktail (mg/m^2)				
# Patients	d1	d3	d5	d7	Total
1	2	2	4	4	12
2	4	4	4	4	16
2	8	8	8	8	32
2	10	10	10	10	40

Appendix I: Flow-chart of IT-cocktail study (summary of study requirements):

PROTOCOL FLOW SHEET
(days following initiation of the study)

	Pre	d1 (1st inj. ↓)	d2	d3 (2nd inj. ↓)	d4	d5 (3rd inj. ↓)	d6	d7 (4th inj. ↓)	d8	d9	d10	d22	d29	d36	d43
Informed consent	o														
History and Phys. exam.	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Chest x-ray	o														
ECG	o														
Vital signs		o[1]	o	o[1]	o	o[1]	o	o[1]	o	o	o	o	o	o	o
Biochemistry[2]	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Hematology[3]	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o[4]
Pharmokinetics		o[5]		o[5]		o[5]		o[5]							
HAMA/HARA	o								o		o	o	o	o	o
Flowcytometry[6]	o		o		o		o		o	o	o	o	o	o	o
Cytokine levels[7]	o		o		o		o		o	o	o	o	o	o	o
Allo-precursors	o								o						
GVHD staging	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o[8]

1: Vital signs are checked every 15 min during the 1st hour post-injection, every 30 min during the 2nd up to the 4th hour, and from then on every hour up to 8 hours post-injection.

2: The pre-study biochemistry panel includes Na^+, K^+, Cl^-, HCO_3^-, urea, creatine, billirubin, glucose, AP, ASAT (GOT), ALAT (GPT), γGT, LDH, and total protein plus electrophoresis.

During the follow-up study, blood will be analyzed daily for Na^+, K^+, Cl^-, HCO_3^-, urea, creatine, glucose,and albumin. Besides, every two days is added billirubin, AP, ASAT (GOT), ALAT (GPT), γGT, LDH, and total protein plus electrophoresis.

3: The hematology panel includes leukocytes plus differentiation, red cells, hemoglobin, hematocrit, thrombocytes.
4: To be continued weekly until WBC numbers have returned to normal.
5: Venous blood samples are obtained pre-injection and 1, 3, 4, 8, 12, 24, and 48 h after each injection. Besides, a sample is taken 72 h following the last injection.
6: The flowcytometry panel includes the markers CD2, CD3, CD4, CD5, CD7, CD8, CD14, CD19 and CD56.
7: Serum is assayed for levels of IL-2, TNF-α and IFN-γ.
8: To be continued monthly when responses are observed.

Appendix II: staging acute graft-versus-host disease

(Glucksberg et al, Transplantation 1974, 18: 295-304)

Skin

grade 1: maculo-papular eruption involving less than 25% of the body surface

grade 2: maculo-papular eruption involving less than 25%to 50% of the body surface

grade 3: generalized erythroma

Liver

grade 1: bilirubin 34-50 µmol/l

grade 2: bilirubin 51-100 µmol/l

grade 3: bilirubin 101-254 µmol/l

grade 4: ³255 µmol/l

Gut

grade 1: 500 to 1000 ml of stool/day

grade 2: 1001 to 1500 ml of stool/day

grade 3: 1501 to 2000 ml of stool/day

grade 4: >2000 ml of stool/day

Overall severity of aGVHD

grade 1: skin: 1/2; gut: 0; liver 1

grade 2:skin: 1/2/3/; gut 1/2 and/or liver 1/2 sometimes associated with fever

grade 3 skin: 2/3/4; gut 2/3/4 and or liver 2/4 often associated with fever.

grade 4: simular to grade 3 but extreme constitutional symptoms

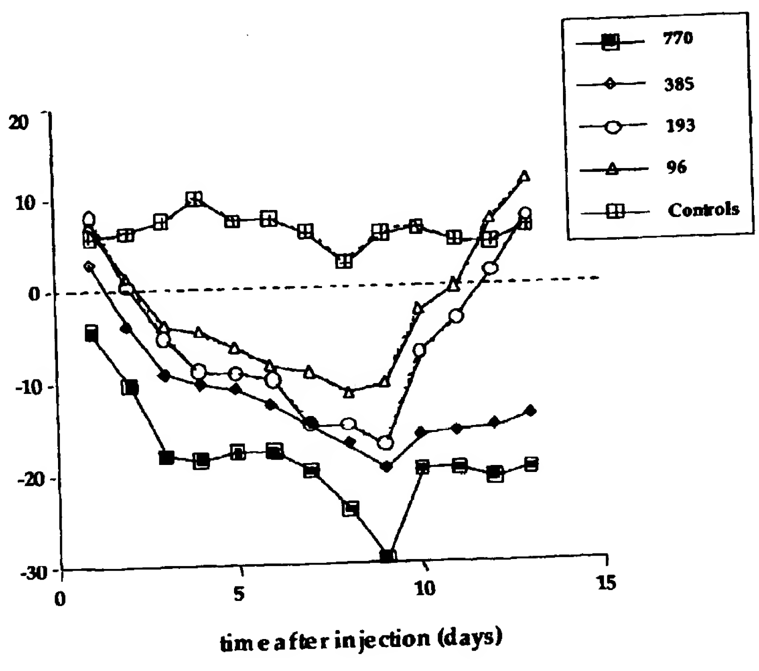
Appendix III

Determination of LD50:

Survival Balb/C mice following i.v. administration IT-cocktail

Dose (μg/mouse)	Time of death	Survival after 2 weeks
770 (n=4)	day 0, day 9, day 9	25%
385 (n=3)	-	100%
193 (n=4)	-	100%
96 (n=4)	-	100%

percentage weight change following administration



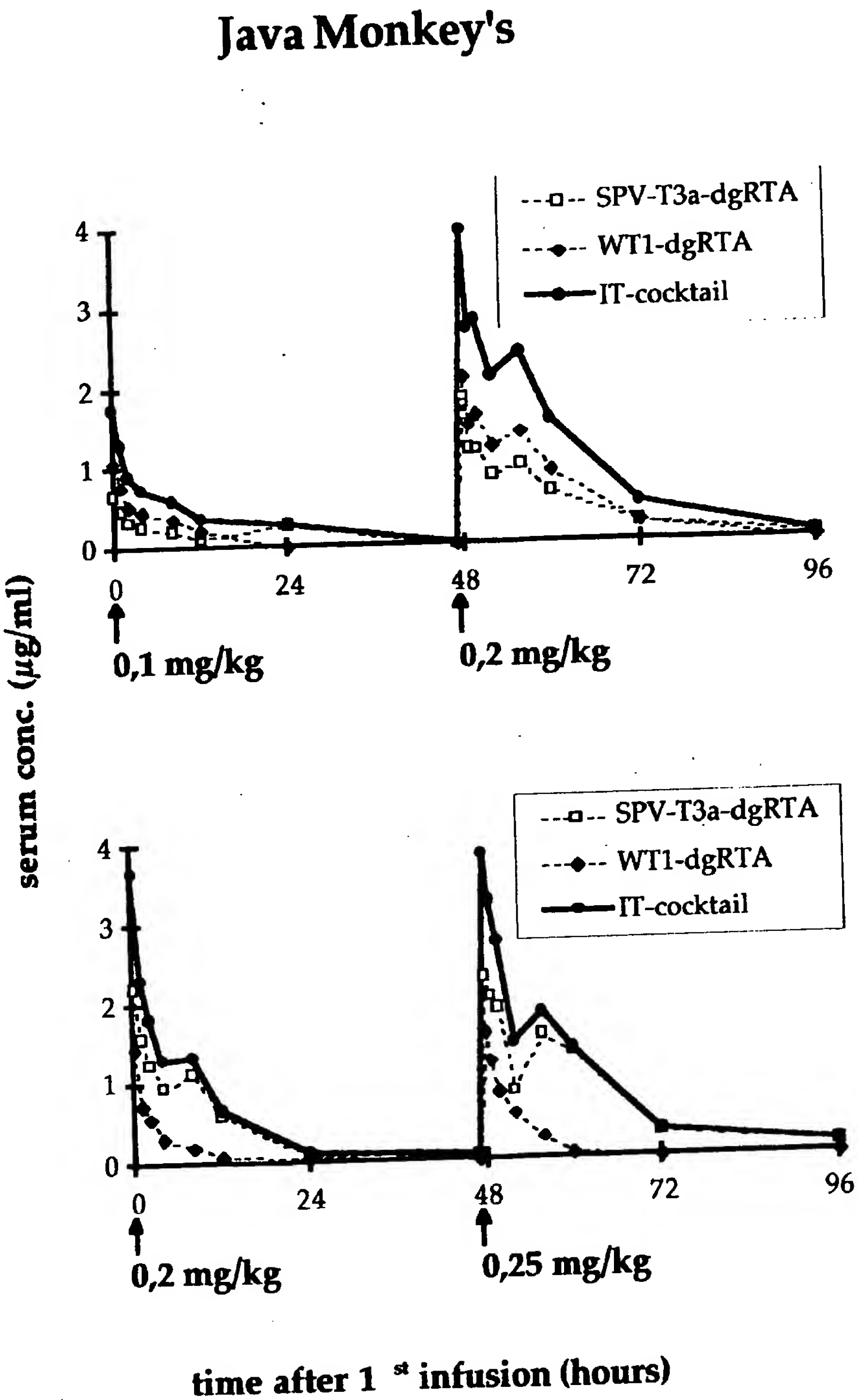
Animal toxicity studies:

A. LD50 determination with Balb/C mice:

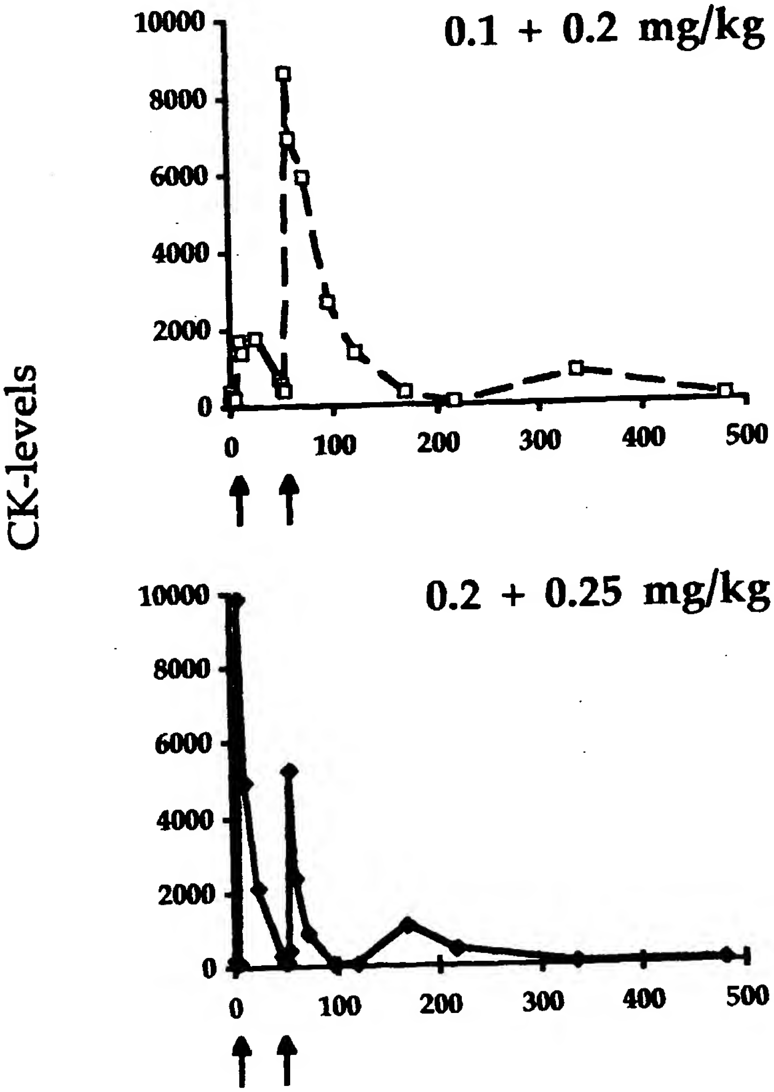
- IT-cocktail: 25-45 mg/kg
- RFB4-dgA: 14 mg/kg

B. Administration to Java-monkey's:

- Rise of CK-levels following infusion
- No further acute toxicities


Java Monkey's
SPV-T3a-dgRTA
WT1-dgRTA
IT-cocktail
4
3
2
1
0
0
24
48
72
96
0,1 mg/kg
0,2 mg/kg
serum conc. (µg/ml)
SPV-T3a-dgRTA
WT1-dgRTA
IT-cocktail
4
3
2
1
0
0
24
48
72
96
0,2 mg/kg
0,25 mg/kg
time after 1 st infusion (hours)

Java-monkey's



time after 1st infusie (hours)

Appendix IV

FIRST PATIENT:

- Male, 60 years, transplanted for MM

- Grade IV GVHD several weeks following aBMT

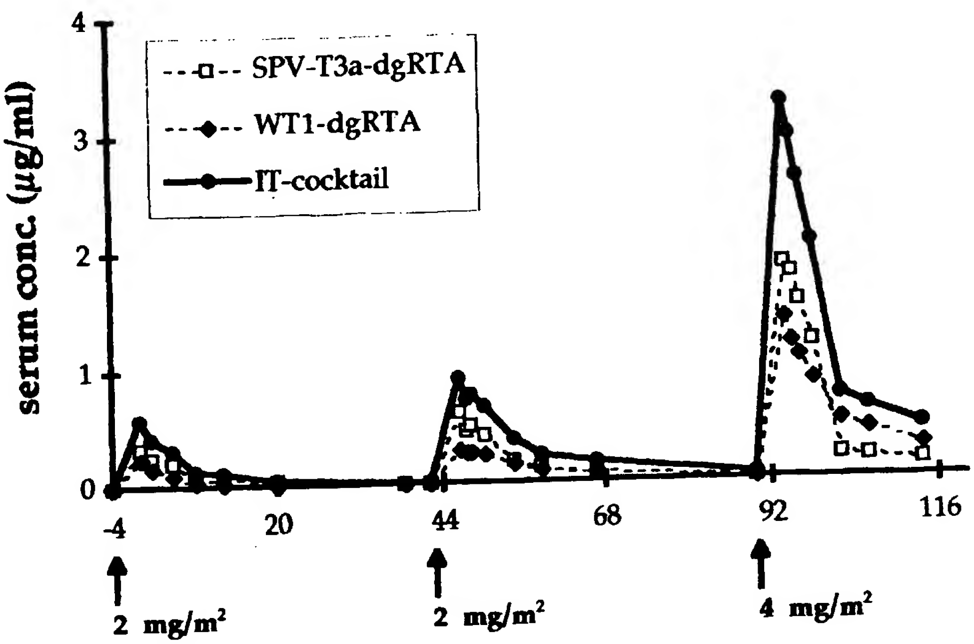
- No response to high dose Solumedrol

- Multi-organ failure

- IT-cocktail: 2 doses 2 mg/m^2, 1 of 4 mg/m^2

- Died eight day's following the first infusion

SERUM CONCENTRATION



- peaklevel IT-cocktail: 0.6, 0.9, and 3.2 μg/ml
- $t_{1/2}$ SPV-T3a-dgRTA: 8,6 uur
- $t_{1/2}$ WT1-dgRTA: 10,4 uur

TOXICITY'S

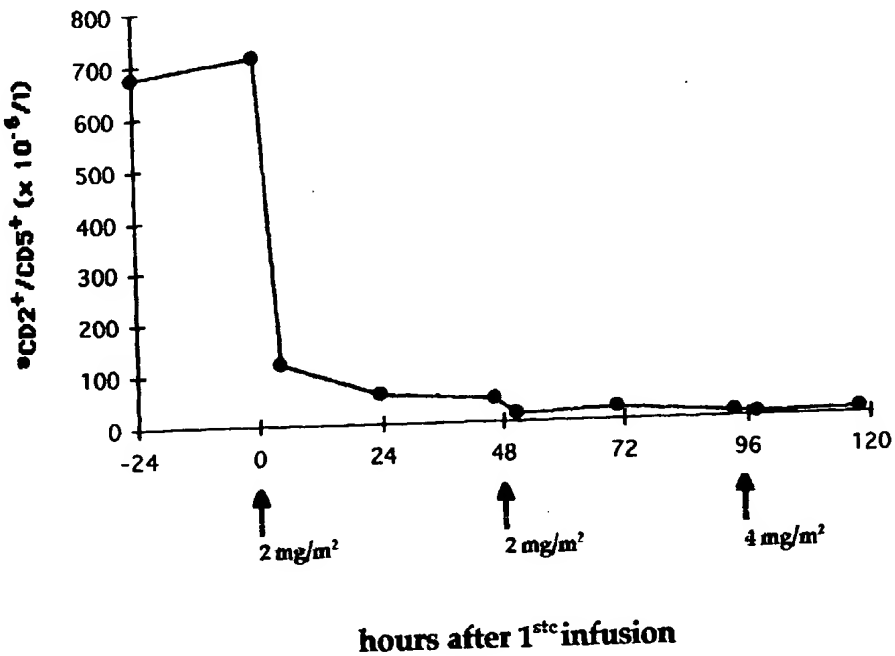
- Mild capillary leakage, no weight gain
- No increase of CK-levels
- No further acute toxicity's

CLINICAL RESPONSE

- Skin: improvement starting at day 5
- Liver: stable (poor condition)
- Gastrointestinal tract: not interpretable

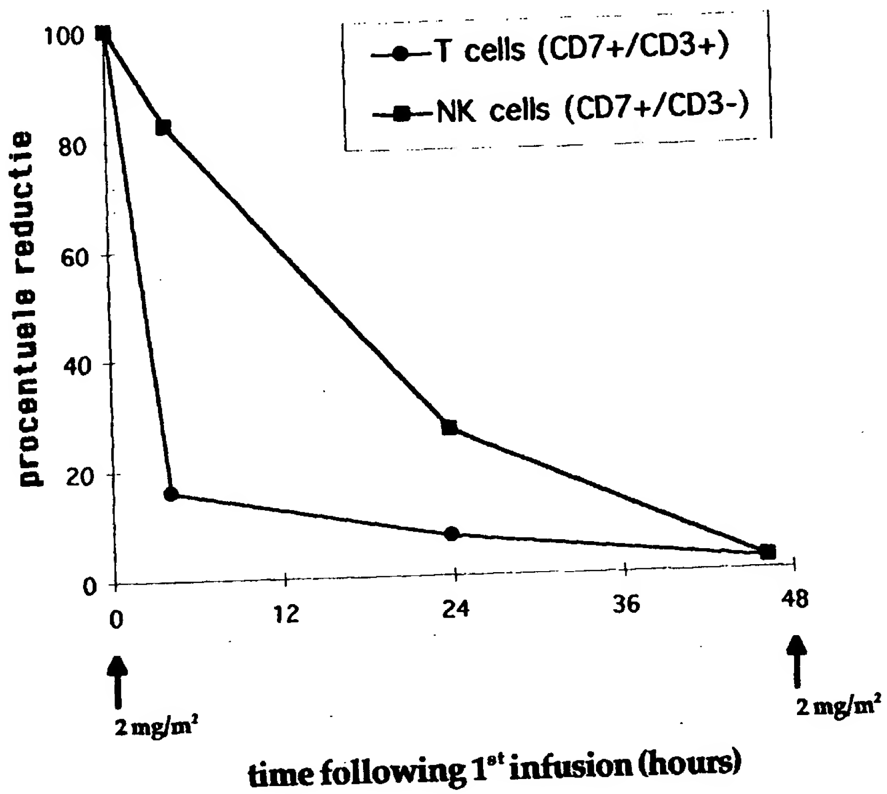
BIOLOGICAL RESPONSE

reduction of lymphocytes



- Fore infusion: 0.7 x 10^9/l circulating T/NK-cells
- Directly following infusion decrease to 17%
- Gradually declines further to 1% at day 7
- Dual mechanism: mAb-based/dgRTA-based

Elimination T cells/NK cells



SECOND PATIENT

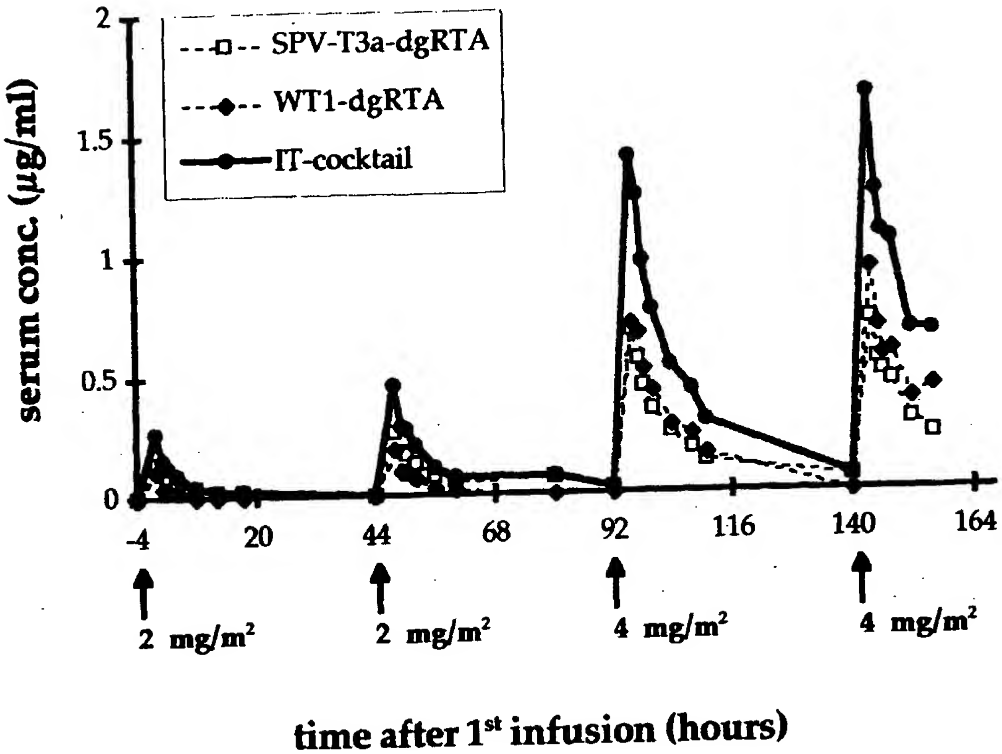
- Male, 34 years, transplanted for CML

- Grade III/IV GVHD of the skin

- No response to high dose Solumedrol

- IT-cocktail: 2 doses 2 mg/m^2, 2 of 4 mg/m^2

Serum concentration IT-cocktail



TOXICITY'S

- No acute toxicity's could be observed

CLINICAL RESPONSE

- Dramatic (complete) response starting at day 5

- Lasting for ~ 1.5 month

- Relapse of GVHD I-II

- Responding to low dose corticosteroids

Claims

1. A pharmaceutical composition for eliminating or reducing the number of unwanted CD3 and/or CD7 positive cells, comprising a mixture of at least a first molecule specifically recognizing CD3 or CD7 and at least a second molecule specifically recognizing another ligand receptor associated with the surface of such an unwanted cell, whereby at least one of the specifically recognizing molecules is provided with a toxic moiety.

2. A pharmaceutical composition according to claim 1, whereby said first molecule specifically recognizes CD3 and said second molecule specifically recognizes CD7.

3. A pharmaceutical composition according to claim 1 or 2, wherein said first molecule is an antibody, or a fragment or a derivative thereof.

4. A pharmaceutical composition according to any one of claims 1-3, wherein said second molecule is an antibody, or a fragment or a derivative thereof.

5. A pharmaceutical composition according to any one of claims 1-4, wherein said toxic moiety is ricin.

6. A pharmaceutical composition according to any one of claim 5, wherein ricin is deglycosylated ricin A.

7. A pharmaceutical composition according to any one of claims 1-6, wherein said toxic moiety is chemically linked to said molecule specifically recognizing CD3, CD7 or another ligand receptor associated with the surface of such an unwanted cell.

8. A pharmaceutical composition according to any one of claims 1-7, wherein at least two molecules specifically recognizing different receptors are provided with toxic moieties, which may be the same or different toxic moieties.

9. A pharmaceutical composition according to any one of claims 1-8, further comprising at least one further molecule specifically recognizing CD5, CD2, CD4, CD8 or an IL-2 receptor.

10. A pharmaceutical composition according to claim 1, wherein said first molecule is a gamma2B IgG antibody or a derivative thereof, which recognizes CD3.

11. A pharmaceutical composition according to anyone of the afore going claims, which comprises at least the equivalent dosis of 25 micrograms of Ricin A per square meter of body surface.

12. A pharmaceutical composition according to claim 11, comprising at least the equivalent dosis of 100 micrograms of Ricin A per square meter of body surface.

13. A pharmaceutical composition comprising at most the equivalent dosis of 25 mg of Ricin A per square meter of body surface.

14. A composition according to anyone of the afore going claims for use as a pharmaceutical.

15. Use of a composition according to anyone of the afore going claims in the preparation of a medicament for the treatment of Graft vs. Host disease, Graft rejections, T-cell leukemias, T-cell lymphomas or other CD3 and/or CD7 positive malignancies, autoimmune diseases or infectious immune diseases such as HIV-infection.

16. A kit of pharmaceutical compositions for treating Graft vs. Host disease and/or graft rejection comprising a composition according to anyone of claims 1-14 and a pharmaceutical composition comprising at least one corticosteroid.

17. A kit according to claim 16 for use as a pharmaceutical.

figure 1: Immunotoxin

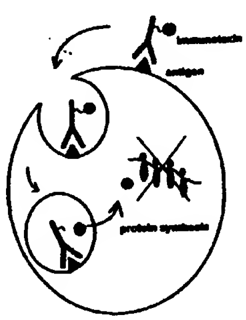


figure 2: Mechanism of action

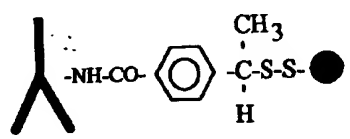


figure 3: SMPT

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 20 0917

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	JANSEN, BURKHARD ET AL: "Successful treatment of human acute T-cell leukemia in SCID mice using the anti- CD7 -deglycosylated ricin A-chain immunotoxin DA7" CANCER RES. (1992), 52(5), 1314-21 CODEN: CNREA8;ISSN: 0008-5472,1992, XP002077287 * table 1 * ---	1-17	A61K47/48
D,X	PREIJERS, F. W. M. B. ET AL: "Relationship between internalization and cytotoxicity of ricin A-chain immunotoxins" BR. J. HAEMATOL. (1988), 70(3), 289-94 CODEN: BJHEAL;ISSN: 0007-1048,1988, XP002077288 * abstract * ---	1-17	
X	PREIJERS, F. W. M. B. ET AL: "Human T lymphocyte differentiation antigens as target for immunotoxins or complement-mediated cytotoxicity" SCAND. J. IMMUNOL. (1988), 28(2), 185-94 CODEN: SJIMAX;ISSN: 0300-9475,1988, XP002077289 * page 188, column 2, paragraph 1; table 1 * ---	1-17	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K
D,X	PREIJERS, F. W. M. B. ET AL: "Different susceptibilities of normal T cells and T cell lines to immunotoxins" SCAND. J. IMMUNOL. (1988), 27(5), 533-40 CODEN: SJIMAX;ISSN: 0300-9475,1988, XP002077290 * page 535, column 2, paragraph 1; figure 1A * --- -/--	1-17	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	11 September 1998	Berte, M

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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EUROPEAN SEARCH REPORT

Application Number
EP 98 20 0917

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	VAN OOSTERHOUT Y V J M ET AL: "Suitability of a cocktail of CD34 and CD7 ricin A-immunotoxins for in vivo treatment of acute graft-versus-host-disease." THIRTY-NINTH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, SAN DIEGO, CALIFORNIA, USA, DECEMBER 5-9, 1997. BLOOD 90 (10 SUPPL. 1 PART 2). 1997. 376B. ISSN: 0006-4971, XP002077291 * page 376B, column 2, paragraph 4439 *	1-17	
A	US 5 045 451 A (UHR JONATHAN W ET AL) 3 September 1991 * claims 1,4,9 *	1-17	
X	WO 89 06967 A (XOMA CORP) 10 August 1989 * claims *	1-17	
X	EP 0 336 379 A (ONCOGEN) 11 October 1989 * claims 1,10 *	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	P. HERVE ET AL.: "Depletion of T-Lymphocytes in Donor Marrow wit PAN-T Moabs and complement for prevention of acute graft-Versus-Host Disease: A Pilot Study on 29 Patients." J. NAT. CANCER INST., vol. 76, no. 6, June 1986, pages 1311-1316, XP002077292 * abstract *	1-17	

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